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Biology of Adenovirus Vectors with E1 and E4 Deletions for Liver-Directed Gene Therapy

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Recombinant adenoviruses with E1 sequences deleted efficiently transfer genes into a wide variety of target cells. Antigen- and nonantigen-specific responses to the therapy lead to toxicity, loss of transgene expression, and difficulties with vector readministration. We have created new cell lines that allowed the isolation of more disabled adenovirus vectors that have both E1 and E4 deletions. Studies with murine models of liver-directed gene therapy indicated that the E1- and E4-deleted vector expresses fewer virus proteins and induces less apoptosis, leading to blunted host responses and an improved safety profile. The impact of the E4 deletion on the stability of vector expression was confounded by immune responses to the transgene product, which in this study was β-galactosidase. When transgene responses were eliminated, the doubly deleted vector was substantially more stable in mouse liver than was the E1-deleted construct. These studies indicate that adenovirus vectors with both E1 and E4 deletions may have advantages in terms of safety and efficacy over first-generation constructs for liver-directed gene therapy.

Vectors based on adenoviruses from which the essential genes E1a and E1b are deleted efficiently target hepatocytes when injected into the circulation (17). The utility of this approach has been tested with recombinant genes potentially therapeutic for a variety of disorders, including urea cycle disorders (28, 44), hyperlipidemia (13, 16, 23), hemophilia (4, 20, 32), and α-1-antitrypsin deficiency (17, 19). Problems with self-limiting transgene expression, hepatitis, and difficulties with vector readministration preclude the successful application of adenovirus vectors to chronic disorders such as those listed above (26, 41).

Insight into the mechanisms responsible for the well-documented problems with E1-deleted vectors initially emerged from experiments performed with mice genetically deficient in cellular immunity (i.e., *nu*/*nu* or *RAG-2*$^{-/-}$ mice), in which transgene expression was stabilized relative to that observed in congenic immune-competent strains (41); this observation has been confirmed with multiple experimental models of liver (2, 9)-, lung (40, 42, 48)-, and muscle (1, 6, 33)-directed gene transfer. Our original interpretation of vector persistence in immune-deficient mice involved either virus replication and reinfection of hepatocytes or persistence of the nonintegrated vector genome in the absence of destructive immune responses to vector-infected hepatocytes. Further disabling the E1-deleted vector by incorporating a temperature-sensitive mutation into E2a had no effect on the duration of transgene expression in the immune-deficient mice, while expression of the transgene was prolonged in immune-competent mice (8, 42). These results suggested that replication-reinfection cannot explain the persistence of this nonintegrating vector. A more likely explanation is that the vector genome is inherently stable; however, the cell is eliminated or the vector genome is destabilized when destructive host immune responses are elicited.

A significant effort has been undertaken to characterize the nature of immune responses to adenoviral vectors. In the liver, E1-deleted vectors express viral antigens that activate cytotoxic T lymphocytes (37, 39, 43); similar responses to reporter gene products that are foreign to the host are likely evoked (33, 34, 39). Activation of T-helper and B cells to viral capsid proteins leads to neutralizing antibodies that block efficient readministration of virus (23, 46). Other factors that may contribute to the type of responses elicited by the host include genotype and major histocompatibility complex haplotype of the recipient, target organ, dose and route of administration of vector, and structure of the vector.

In this study, we describe isolation of cell lines useful in generating adenovirus vectors based on human adenovirus type 5 (Ad5) from which both E1 and E4 were deleted. Several groups have recently described similar cell lines, although in vivo studies of the resulting vectors have not been presented (25, 35, 45). The biology of this new generation of vectors was studied in murine models of liver-directed gene therapy.

MATERIALS AND METHODS

Construction of E4 ORF6 plasmids. E4 open reading frame 6 (ORF6) sequence was amplified by anchored PCR and was cloned into the pBS(+) vector. The following oligonucleotide primers were used: E4 ORF6 sense primer (5′-ATGACTACGTCCGGCGTT-3′ [bp 34078 to 34061]) and E4 ORF6 antisense primer (5′-AAGCTTGGATCCCTACATGGGGGTAGAGTC-3′ [bp 33194 to 33211]). The ORF6 fragment in pBS(+)ORF6 was sequenced and subcloned into plasmids that contained the neomycin resistance gene for G418 selection and allowed expression from either the zinc-inducible sheep metallothionein promoter (pMT-ORF6) or the mouse mammary tumor virus (MMTV) promoter (pMMTV-ORF6).

Transfections and selection of G418-resistant clones. 293 cells were obtained from the American Type Culture Collection and were maintained as monolayers in Dulbecco's modified Eagle's minimal essential medium supplemented with 10% fetal bovine serum. Plasmids were transfected by calcium phosphate precipitation onto 293 cells seeded in 100-mm plates, with 10 μg of plasmid DNA for each plate. Twenty-four hours posttransfection, the cells were trypsinized and seeded in G418-containing media at various dilutions ranging from 1:5 to 1:40. After 2 weeks of selection, G418-resistant colonies were isolated, expanded, and screened for E4 expression.

Screening procedure for E1-E4 cell lines. Stable G418-resistant clones were first screened with a recombinant adeno-associated virus (rAAV)-LacZ transduction enhancement assay in which 5×10^4 cells in 96-well plates were infected with 3×10^6 genome copies of rAAV-LacZ with and without inducers. Twenty-four hours postinfection, the cells were histochemically stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), and the percentage of blue cells in each well was scored. Subsequently, the strongly positive clones based on the

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primary screen were infected with an E4-deleted mutant virus, *dl*1004 (3), at a multiplicity of infection (MOI) of 0.1 for 24 h. The abilities of cell lines to complement adenovirus late gene expression were assessed by immunofluorescence staining with a rabbit polyclonal antibody to Ad5 capsid proteins. As a final screen, the positive clones selected from previous steps were infected with *dl*1004 virus over a range of serial dilutions and after 20 h were overlaid with top agar consisting of 0.8% agar noble (Difco), 2× basal Eagle's medium (Gibco), 12.4 mM $MgCl_2$, and 2% fetal bovine serum. For cells containing MT-ORF6 or MMTV-ORF6 minigenes, induction was achieved with 150 μM $ZnSO_4$ or 10 μM dexamethasone (DEX), respectively. The ability to support plaque formation by these clones was assessed by staining with neutral red on day 10 postinfection.

Construction of recombinant adenovirus vector with E1-E4 double deletions. The E1-E4 doubly complementing cells were seeded in 60-mm plates and cotransfected with *Cla*I-digested *dl*1004 viral DNA and *Nhe*I-digested pAdCBLacZ plasmid DNA (2 μg of viral DNA and 10 μg of plasmid DNA for each plate) by the calcium phosphate precipitation method. The pAdCBlacZ plasmid contained the 5′ region of Ad5 (map units 0 to 16) with sequences spanning E1a and E1b that had been deleted (map units 1 to 9.6) and replaced with a *lacZ* minigene driven by the chicken β-actin promoter with enhancer sequences from the cytomegalovirus immediate-early gene sequences (22). Twenty hours posttransfection, the cells were overlaid with top agar. Well-isolated plaques were picked at 10 days posttransfection following neutral red staining, and were screened for recombinant viruses generated by homologous recombination by the X-Gal histochemical staining method. After three rounds of plaque purification, the recombinant viruses were amplified on E1- and E4-expressing cells and purified according to standard protocols (22). Measurement of A_{260} (10^{12} virions per 1 absorbance U) was used to estimate virus concentration. This vector was called H5.001CBlacZ, on the basis of the nomenclature used to describe vectors generated at the Institute for Human Gene Therapy at the University of Pennsylvania (8).

Characterization of E1-E4 doubly complementing cell lines. (i) Genetic constitution. The genomic DNAs prepared from E1-E4 MT-ORF6 (10-3) and E1-E4 MMTV-ORF6 (27-18) cells and the plasmid DNAs used for generating these cell lines were digested with appropriate restriction endonucleases to release the E4 ORF6-containing fragment. DNAs were fractionated on 1% agarose gels, transferred to nylon filters, and hybridized with an ORF6 DNA probe.

(ii) E4 ORF6 protein production. Cell pellets of 10-3 and 27-18 cells were harvested from 60-mm plates and resuspended in 200 μl of lysis buffer (20 mM Tris-Cl [pH 8.0], 140 mM NaCl, 1% Nonidet P-40 [vol/vol], 1 mM phenylmethylsulfonyl fluoride, 1 μg each of leupeptin, antipain, chymostatin, and soybean trypsin inhibitor per ml). The lysates were incubated on ice for 1 h and spun in an Eppendorf microcentrifuge at 14,000 rpm for 30 min at 4°C. The supernatants were collected, and total protein concentrations were determined by Lowry's method. Samples (50 μg) were fractionated on sodium dodecyl sulfate-12.5% polyacrylamide gel electrophoresis (SDS-PAGE) gels and electrotransferred to nitrocellulose membranes. E4 ORF6 protein was detected with the ECL system (Amersham Life Science) with a mouse monoclonal antibody produced against an ORF6 bacterial fusion protein (31). For the MMTV-ORF6 cell line (27-18), cells were prepared with or without a 24-h induction in 10 μM DEX. The MT-ORF6 cells (10-3) were sampled with or without a 24-h induction by 150 μM $ZnSO_4$. 293 cells infected with wild-type adenovirus and W162 cells (36) either uninfected or infected with *dl*1004 virus were used as controls.

(iii) RPEs for *dl*1004 virus on the E1-E4 cell lines. E1-E4 ORF6 cells were compared with 293 and W162 cells in their abilities to support plaque formation of *dl*1004 virus. The cells were infected with *dl*1004 virus over a range of serial dilutions and were overlaid with top agar 20 h later. DEX (10 μM) was used for induction of 27-18 cells during the entire infection and plaque formation process. For the cell line 10-3, $ZnSO_4$ (150 μM) was added to the medium 24 h before and during the infection. Plaques were detected on day 10 postinfection, and the relative plaquing efficiency (RPE) was computed as the ratio of *dl*1004 titer for the cell line to that measured for W162 cells.

(iv) Growth kinetics of an E1-E4-deleted recombinant virus H5.001CBLacZ on the doubly complementing cell lines. E1-E4-expressing 293 cells were infected with H5.001CBLacZ at an MOI of 0.5. Inducers (150 μM $ZnSO_4$ for 10-3 cells or 20 μM DEX for 27-18 cells) were added to the media 24 h before the infection and maintained during the infection. The infected cells were harvested various times (i.e., 24 to 72 h) postinfection and lysed in infection medium by three rounds of freezing-thawing, and the titer of virus in each lysate was determined by infections with serial dilutions on 293 cells and then by histochemical staining with X-Gal. Titers were expressed as *lacZ*-forming units (LFU) per milliliter, where 1 LFU was defined by the foci of *lacZ* transduction in a limiting-dilution infection analyzed 24 h after the infection was initiated.

Animal studies. The mice used in this study were C57BL/6 (6 to 8 week-old females), *nu*/*nu* in a BALB/C background, C3H, or ROSA-26 in a C57BL/6-129 chimeric background. The mice were instilled with H5.CBlacZ (a vector with only E1 deleted) or H5.001CBlacZ via the tail vein (10^{11} particles in 100 μl of phosphate-buffered saline) on day 0, and necropsies were performed at days 3, 10, and 60; lung or liver tissues were prepared for cryosections on days 3 and 60, while spleen or mediastinal lymph nodes were harvested for immunological assays on day 10. Infusions were normalized to particles, since comparison of titers based on PFU between an E1-deleted versus an E1- and E4-deleted vector was difficult, because it depends on the cell line on which the cells are plaqued. The efficiency of transduction based on β-galactosidase expression in the infected cells was not significantly different in vitro when equivalent numbers of particles were used (data not shown).

Morphological assays. Liver tissues were frozen in Tissue-Tek Optimal Cutting Temperature compound (Miles Inc., Elkhart, Ind.), cryosectioned, and analyzed for β-galactosidase activity with specific histochemical stains and for viral late proteins by using immunofluorescence staining as previously described (37, 41). In addition, liver sections were subjected to in situ hybridization analysis for the expression of vector-derived *lacZ* mRNA with RNA probes to simian virus 40 poly(A), which is unique to adenovirus-derived *lacZ* RNA. In situ detection for apoptotic hepatocytes was also performed on the liver cryosections with Apop Tag Plus following the manufacturer's instructions (Oncor).

Histopathology. Liver tissues were harvested, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. A scoring system for grading vector-induced hepatitis based on four criteria was developed as follows: portal inflammation, 10 portal triads graded from 0 (normal) to 3 (severe) and added for a maximum of 30; lobular inflammation, same as for portal inflammation; apoptosis, apoptotic figures identified based on morphologic criteria and counted over 10 fields at a magnification of ×40; and mitosis, total number of cells undergoing mitosis counted over 10 fields at a magnification of ×40.

Immunological assays. Lymphocytes from spleens or mediastinal lymph nodes were restimulated in vitro with virus and used for CTL and proliferation assays as described elsewhere (37).

RESULTS AND DISCUSSION

Isolation of an adenovirus vector with E1 and E4 deletions. The first step in creating a vector with the essential genes E1 and E4 deleted was to isolate a transcomplementing cell line. The general strategy was to transfect into 293 cells E4 sequences necessary to complement a full deletion of this region of the virus.

The E4 region of human Ad5 lies between map units 99 and 91.5 and is transcribed off a single promoter from the *l* strand (Fig. 1A). This locus expresses at least seven ORFs, the polypeptides of which have been identified and partially characterized elsewhere (3, 5, 10, 14, 15, 21, 27, 29, 30). When developing a cell line that complements an E4 deletion, we took into account the fact that constitutive expression of E4 is toxic to cells and that a complete deletion of this region can be complemented by expression of either ORF3 or ORF6 proteins (3). Plasmid-based vectors were created that express E4 ORF6 from promoters inducible by either divalent cations (i.e., sheep metallothionein gene) or with glucocorticoids (i.e., the MMTV long-terminal repeat [Fig. 1B]). There is no overlap between the ORF6 and sequences retained in the *dl*1004-based vector, eliminating the chance of reversion by homologous recombination.

Each ORF6 plasmid was transfected into 293 cells, and individual clones were isolated and characterized. A convenient screening assay for ORF6 expression was developed based on the observation that ORF6 markedly enhances rAAV transduction (10, 11). Clones of 293 cells stably transfected with ORF6 plasmids were infected with purified *lacZ* rAAV and subsequently analyzed by X-Gal histochemistry. The level of transduction was assayed in the presence or absence of DEX as an index of ORF6 induction; four clones substantially enhanced rAAV transduction in the presence of DEX (data not shown). Two additional assays confirmed with complete concordance clones expressing the highest levels of transcomplementing ORF6 protein. These assays were based on expression of late viral genes or formation of plaques after infection with the E4-deleted virus *dl*1004 (data not shown).

On the basis of the criteria of efficient and consistent enhancement of rAAV transduction, a preferred clone from each transfection was identified (i.e., clone 27-18 from the MMTV ORF6 transfection and clone 10-3 from the metallothionein ORF6 transfection) and subjected to further analyses. Additional studies included DNA hybridization for ORF6 sequences (Fig. 2A) and Western blot (immunoblot) analysis for

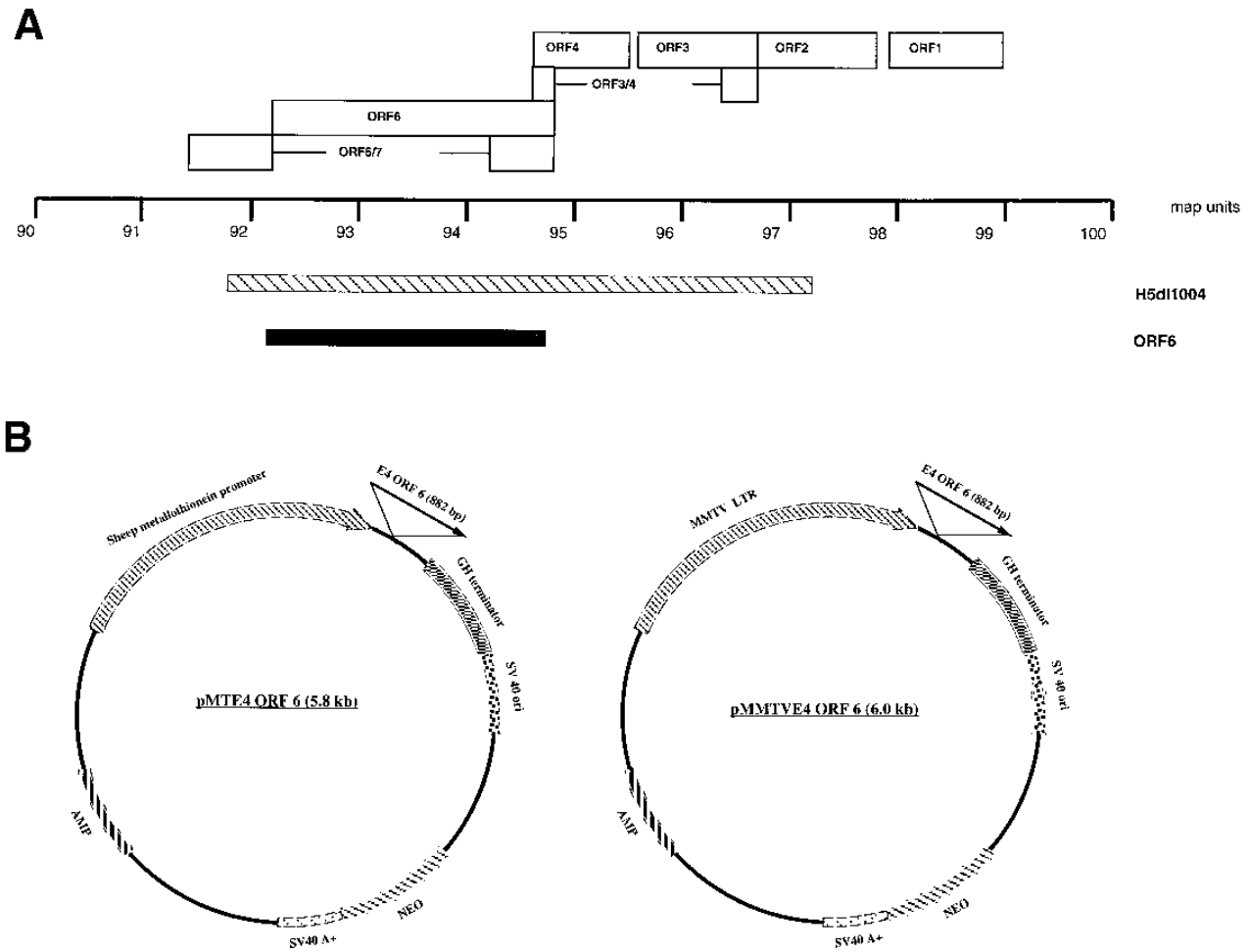

FIG. 1. Schematic representation of the Ad5 E4 region and construction of vectors for generating stable cell lines. (A) ORFs of E4 are indicated by open boxes above the Ad5 genomic map coordinates. The deletion in H5*dl*1004, represented by the striped bar, spans bp 33092 to 34955 (Δ1863 bp). The ORF6 fragment cloned for cell lines, indicated by the solid bar below, spans bp 33194 to 34078 (Δ884 bp). (B) Plasmids expressing ORF6. GH, growth hormone; AMP, ampicillin resistance gene; NEO, neomycin resistance gene; LTR, long-terminal repeat; SV40, simian virus 40.

the ORF6 protein (Fig. 2B). Analysis of total cellular DNA revealed approximately 1 copy of plasmid DNA per cell stably integrated into the genome of both 27-18 and 10-3 cell lines. Without induction, ORF6 protein was not detectable by Western analysis in either cell line; following induction, significant levels of ORF6 were detected in both 27-18 and 10-3 cells, with the highest levels achieved in 10-3 cells induced with zinc. The induced protein, which migrated at an apparent molecular mass of 34 kDa, was identical to that detected in 293 cells infected with wild-type Ad5 and W162 cells infected with *dl*1004 (Fig. 2B). With induction, the cells stopped proliferating, in keeping with the presumed toxic effects of ORF6. The mechanism of this apparent toxicity is unknown, although it may involve effects on the cell cycle, since cells accumulate in S phase following induction of ORF6 (data not shown).

Critical to the use of these cell lines for isolating recombinant vectors is their ability to plaque E4-deleted virus. Monolayers of cells were infected with serial dilutions of *dl*1004 and overlaid with agar to estimate plaquing efficiency. Early-passage 10-3 and 27-18 clones were capable of plaquing *dl*1004 virus at efficiencies higher than that achieved with the standard cell line W162 (Fig. 3A). The RPEs of both cell lines were relatively stable following sequential passage up to passage 20 (Fig. 3A).

A *lacZ*-containing vector with both E1 and E4 deleted was isolated with the 27-18 cell line. The previously described E4 mutant *dl*1004 was used in these constructions; this virus had all E4 ORFs deleted, except ORF1, which was intact (Fig. 1A). *Cla*I-restricted *dl*1004 was cotransfected with the linearized *lacZ* adenovirus vector, and resulting plaques were expanded on 27-18 cells. After three sequential plaque purifications, the stocks of vector were shown to be free of virus containing either E1 or E4. This new virus, called H5.001CBLacZ, was grown on the two candidate cell lines 27-18 and 10-3 to quantify yield of virus, measured as the amount of LFU generated as a function of time after infection (Fig. 3B). No E1-E4-deleted *lacZ* virus is propagated on 293 cells, indicating the absence of any detectable reversion of the E4 deletion. Both early- and late-passage 27-18 and 10-3 cells yielded similar quantities of the E1-E4-deleted vector.

Toxicity in mouse liver is reduced with vectors with both E1 and E4 deleted. The hypothesis for this study was that a vector with both E1 and E4 deleted would express lower levels of early and late viral proteins, leading to a decrease in toxicity because of blunted antigen- and nonantigen-specific immune responses. The result should be a less-immunogenic vector that confers more-stable expression.

The toxicity of adenovirus vectors injected into the periph-

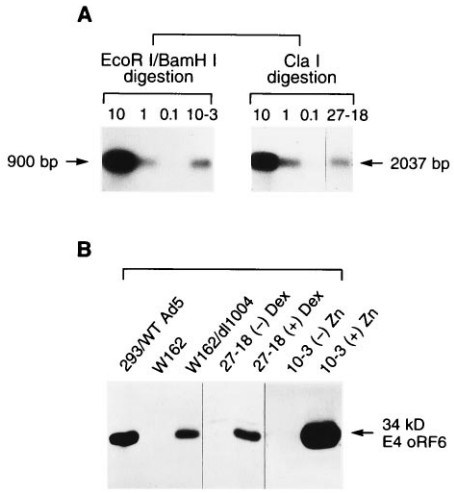

FIG. 2. (A) Southern blot of genomic DNA prepared from 10-3 (MT-ORF6) and 27-18 (MMTV-ORF6) cells. High-molecular-mass DNAs from 10-3 or 27-18 cells were digested with the restriction endonuclease(s) denoted above each panel, fractionated on a 1% agarose gel, transferred to a nylon filter, and hybridized with an ORF6 DNA probe. The left three lanes contain plasmid DNAs equivalent to 10, 1, or 0.1 copies per diploid cell for pMTORF6 and pMMTVORF6. (B) Western blot analysis of ORF6 protein expressed in E1-E4 doubly complementing cell lines. Cellular lysates were prepared and fractionated on an SDS-12.5% PAGE gel, with 50 μg of protein loaded per lane. Proteins were electrotransferred to a nitrocellular membrane, and ORF6 was detected with a mouse monoclonal antibody by using the ECL system. Control samples included 293 cells infected with wild-type Ad5 virus and W162 cells either uninfected or infected with H5*dl*1004 virus. When appropriate, cells were sampled with and without induction (with DEX for 27-18 cells and $ZnSO_4$ for 10-3 cells).

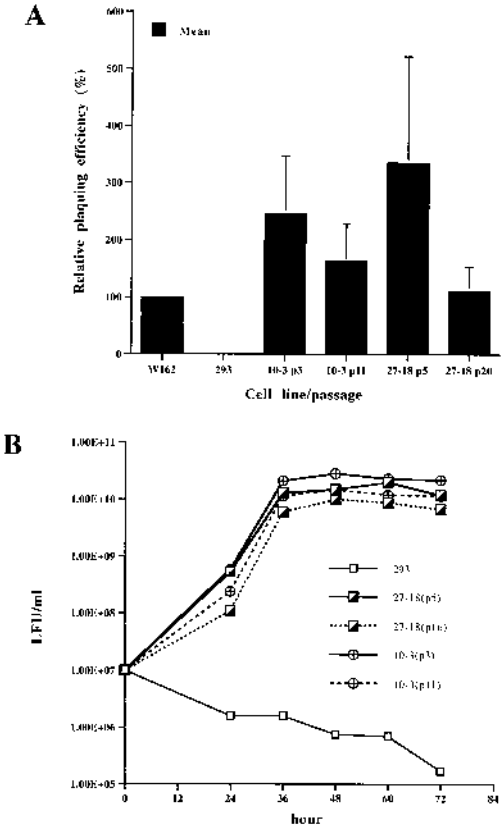

FIG. 3. (A) RPEs for an E4-deleted virus on the E1-E4 double complementation cell lines and cell line stabilities over passages. 10-3 and 27-18 cells from different passages were compared with 293 and W162 cells. Cells were infected with *dl*1004 virus over a range of serial dilutions and were overlaid with top agar after 20 h. DEX (10 μM) was added to both the infection media and top agar for the 27-18 cells containing MMTV-ORF6. For the cell line 10-3 with the MT-ORF6 cassette, $ZnSO_4$ (150 μM) was added to the medium 24 h before infection and during the infection but was absent from the top agar. Plaques were detected by staining with neutral red on day 10 postinfection. RPEs were computed as the percentage of the titer of *dl*1004 compared with W162 cells. Solid bars, the mean RPE of each cell line from three independent experiments; error bars, standard deviations. (B) Growth kinetics of an E1-E4-deleted recombinant virus, H5.001CBLacZ (see Materials and Methods), in 10-3 and 27-18 cells from different passages. The cells were infected with H5.001CBLacZ virus at an MOI of 0.5 with induction (150 μM $ZnSO_4$ for 10-3 cells and 20 μM DEX for 27-18 cells). The infected cells were harvested at 24, 36, 48, 60, and 72 h postinfection. The cells were lysed in the infection medium by three rounds of freezing-thawing, and virus titers on 293 cells were determined by infections with serial dilutions. The cells were histochemically stained with X-Gal after 20 h, and blue cells were counted. The yield of H5.001CBLacZ virus in each cell line is shown on the *y* axis in log numbers. The time points are shown on the *x* axis.

eral circulation is largely confined to the liver, where a biphasic response is observed (18, 37, 41). Within the first 1 to 3 days following gene transfer, a mild neutrophilic infiltrate is observed, as is the presence of apoptotic degeneration of hepatocytes. This is often associated with an increase in liver transaminases in serum. The second phase of inflammation is characterized by mononuclear cell infiltration throughout the lobule and in the periportal area associated with a second peak of elevated serum transaminase levels.

Several different strains of mice were infused with 10^{11} particles of the first-generation *lacZ* adenovirus vector, H5.CBlacZ, or the corresponding E1-E4-deleted version, H5.001CBlacZ. Three days later, livers were harvested and analyzed for transduction by X-Gal histochemistry, which demonstrated transgene expression in greater than 90% of the hepatocytes for each vector (data not shown); expression of late viral genes was substantially reduced in livers from animals infected with the E1-E4-deleted vector (example of C3H mice [Fig. 4]).

Liver tissues were analyzed for vector-induced pathology by evaluating paraffin sections at the light-microscopic level with a previously described scoring system which quantifies portal and lobular inflammation and apoptotic and mitotic figures (18). Histochemical data are presented in Fig. 5, while serum transaminase ALT levels (a biochemical measure of liver toxicity) are presented in Table 1. Examples of vector-induced apoptosis are shown in Fig. 4. The picture in C57BL/6 mice was dominated by portal and lobular inflammation, peaking at day 14, with little apoptosis. No significant difference was observed between the two vectors. Serum ALT levels were elevated as early as day 3, peaking at day 14 with both vectors; increases in ALT observed with the E1-E4-deleted vector were two- to fourfold lower than what was noted with the first-generation construct (Table 1). Qualitatively different results were obtained in C3H mice in which apoptosis was the dominant finding and inflammation was mild. These pathological lesions peaked at day 14 and were significantly greater in animals that received the E1-deleted vector than in those with the E1-E4-deleted construct (Fig. 5). Serum ALT levels were also elevated in C3H mice, with greater increases noted with the first-generation vector. The levels of serum ALT, which also peaked at day 14, were lower for both vectors in C3H mice than what was observed for C57BL/6 mice. As expected, inflammatory responses were not seen with either vector in *nu*/*nu* mice (Fig. 5). The only detectable lesion in these animals was apoptosis at day 14, with the E1-deleted vector that was associated with a substantial rise in ALT; no significant histopathology or eleva-

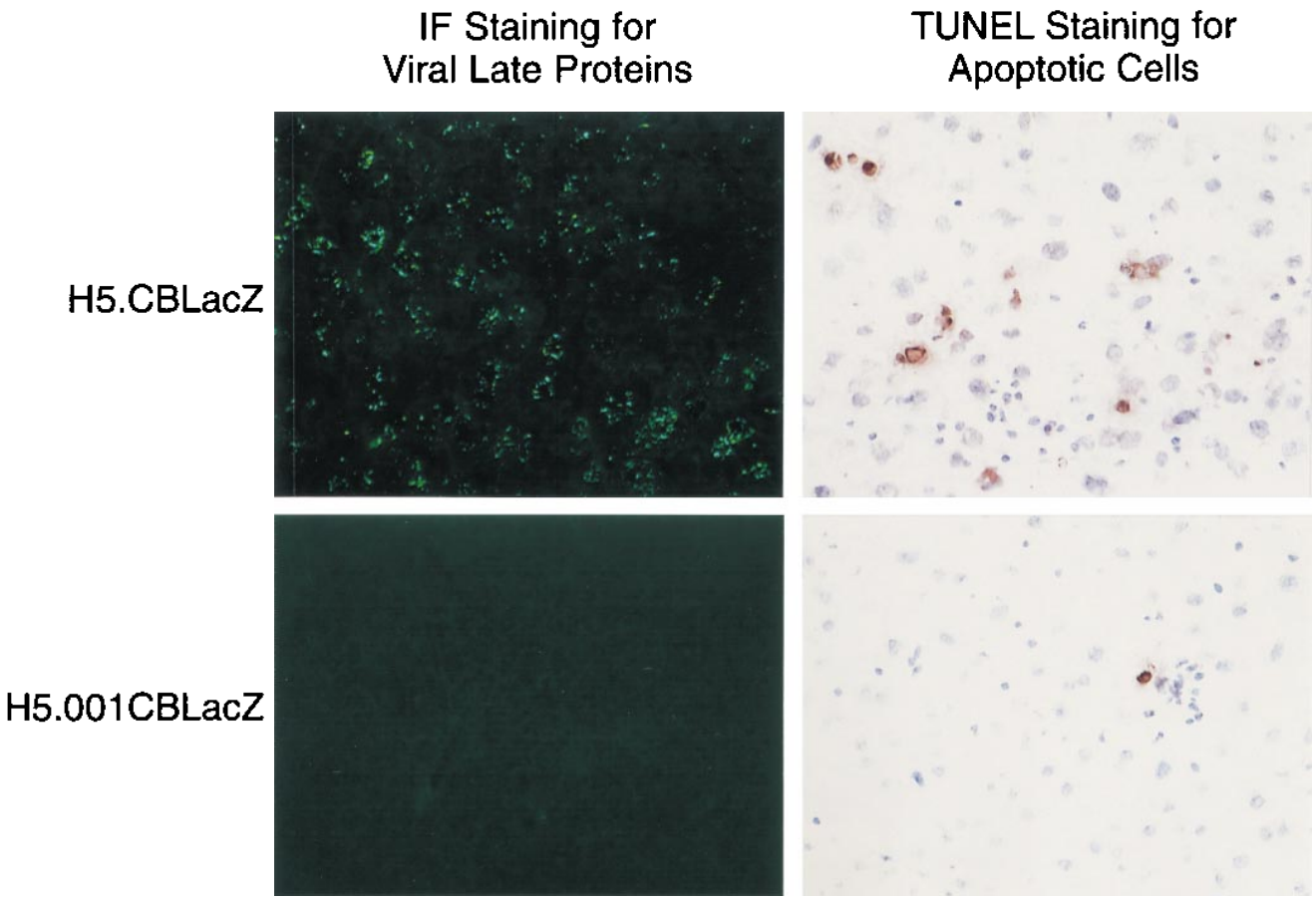

FIG. 4. Viral late protein expression and hepatic apoptosis in C3H mouse livers after infusion of H5.CBLacZ and H5.001CBLacZ viruses. Six- to 8-week-old female C3H mice were intravenously injected with the E1-deleted and E1-E4-deleted viruses (10^{11} particles per animal). Four days after infusion, the mice were sacrificed, and cryosections of liver tissues were prepared. Viral late proteins were detected with a rabbit polyclonal antibody by using immunofluorescence staining, whereas apoptotic hepatocytes were detected with terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling (TUNEL) reaction by using ApopTag Plus (Oncor).

tions in serum transaminase levels were observed in *nu*/*nu* mice infused with the E1-E4-deleted vector (Fig. 5; Table 1).

Deletion of E4 from an adenovirus vector blunts cellular immunity and prolongs transgene expression. Our hypothesis predicts that diminished viral gene expression from the E1-E4-deleted vector should translate to less cellular immunity and more-prolonged gene expression. Vectors were infused into C57BL/6 mice, and lymphocytes were harvested 10 days later for in vitro studies of cytotoxic T lymphocytes (CTLs [Fig. 6]) and 3 and 60 days after gene transfer for efficiency and stability of transgene expression (Fig. 7 [histochemical analysis] and Fig. 8 [in situ hybridization]).

Analysis of lymphocytes from vector-treated mice (i.e., E1-deleted and E1-E4-deleted *lacZ* vector) revealed CTL activity to major histocompatibility complex identical target cells infected with E1-deleted adenovirus expressing either *lacZ* or another reporter gene, ALP (Fig. 6). This is similar to previous reports that demonstrated activation of CTLs to viral proteins (37, 39, 41). CTL activity was noted in lymphocytes of animals treated with either vector, although the total activity of these bulk-cultured, in vitro-stimulated lymphocytes was lower in animals that received the E1-E4-deleted vector than what was observed with lymphocytes from E1-deleted vector-treated animals. Much to our surprise, transgene expression was no more stable with the E1-E4-deleted vector (Fig. 7A and B) than what was observed with the E1-deleted vector (Fig. 7C and D); expression was gone by day 60 (see Table 2 for morphometric analysis).

We considered four hypotheses to explain the results of transgene in stability described above: (i) host responses to virus proteins do not contribute to transgene instability, (ii) the decrease in viral protein expression achieved with the E1-E4-deleted vector is insufficient to blunt host immune responses to a level that would prolong gene expression, (iii) deletion of E4 has destabilized the viral genome independently of immunity, and (iv) immune responses to the transgene target obscure any vector-specific advantages realized by the deletion of E4.

A new experimental paradigm was developed to formally evaluate these hypotheses. Experiments performed with C57BL/6 mice were repeated with *nu*/*nu* animals to study vector stability independently of immunity. Both vectors were stably expressed in *nu*/*nu* mice, indicating that the E4 deletion did not destabilize the genome (E1 deletions [Fig. 7E and F] and E1-E4 deletions [Fig. 7G and H]; morphometric analysis is presented in Table 2). Potentially confounding immune responses to the transgene product were eliminated by performing identical experiments with the ROSA-26 strain of mice, which harbor a β-galactosidase transgene incorporated into the germ line (12). These animals express β-galactosidase in multiple tissues, although the level of transgene expression is

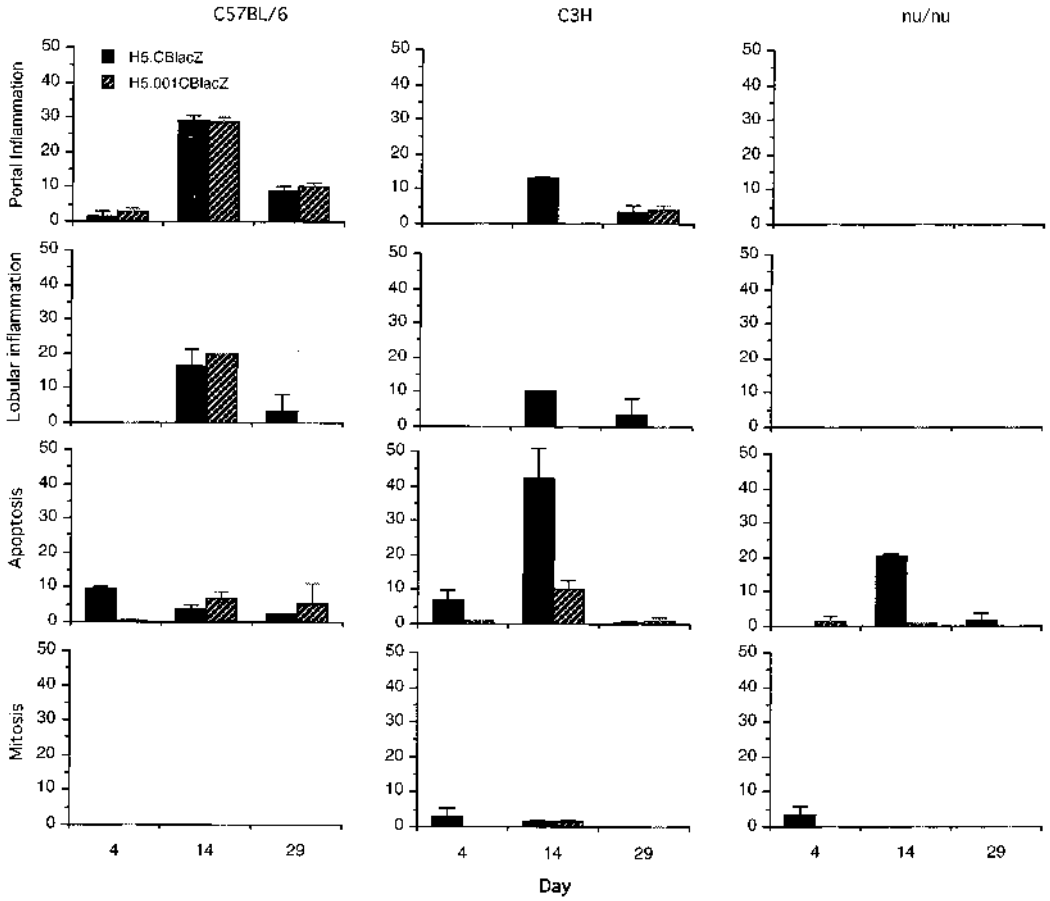

FIG. 5. Evaluation of pathological response of recipient mouse liver to recombinant adenoviruses; pathological response in C57BL/6, C3H, and *nu/nu* mice receiving H5.CBLacZ or H5.001CBLacZ. Liver tissues were harvested at the indicated time points following infusion of E1-deleted (H5.CBLacZ) or E1-E4-deleted (H5.001CBLacZ) recombinant adenovirus (10^{11} particles per mouse) and were evaluated for evidence of histopathology by light-microscopic examination of paraffin sections stained with hematoxylin and eosin. The pathological lesions were characterized in four categories: portal inflammation, lobular inflammation, apoptosis, and mitosis. The scoring system for quantifying the severity in each category was developed specifically for adenovirus vector-related toxicity. The histogram shown is the average of at least three independent observations, with standard errors of the means indicated by error bars.

low in the liver. Initial studies utilized X-Gal histochemistry to detect the adenovirus vector-encoded β-galactosidase, which is expressed at substantially greater levels than the endogenous transgene. Expression of *lacZ* from the E1-deleted vector diminished substantially (i.e., at least 10-fold reduction) over 60 days (Fig. 7I and J; Table 2), although it was slightly more stable than what was observed in C57BL/6 mice (Fig. 7A and B; Table 2). Expression of vector-encoded *lacZ* was stabilized in ROSA-26 mice infused with the E1-E4-deleted vectors, with

TABLE 1. Levels of ALT in serum in mice after viral administration

Days postinfusion and adenovirus vector[a]	ALT activity (mean ± SE [IU/liter])		
	C57BL/6	C3H	*nu/nu*
3			
H5.CBLacZ	878 ± 1,750	426 ± 87	260 ± 295
H5.001CBLacZ	243 ± 353	162 ± 82	62 ± 21
14			
H5.CBLacZ	1,473 ± 834	619 ± 196	1,982 ± 992
H5.001CBLacZ	633 ± 394	285 ± 124	65 ± 30

[a] 10^{11} particles per animal (n = 3).

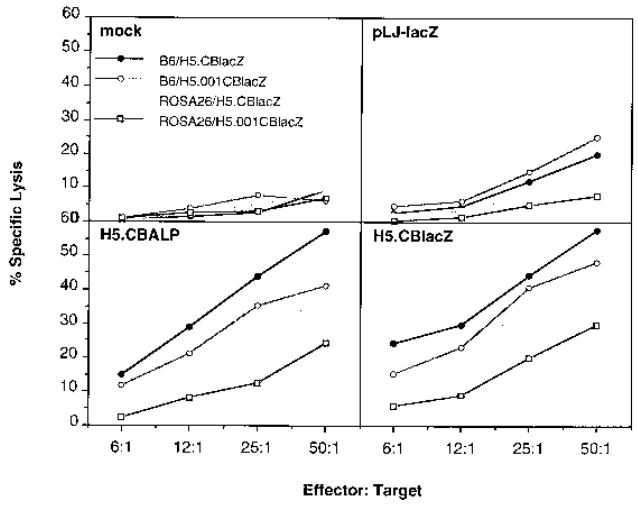

FIG. 6. CTL responses to recombinant adenovirus vectors. Splenocytes harvested from C57BL/6 mice infected with H5.CBlacZ or H5.001CBlacZ and ROSA-26 mice infected with H5.CBlacZ (ROSA26/H5.CBlacZ) or H5.001CBlacZ (ROSA26/H5.001CBlacZ) were restimulated in vitro for 5 days and tested for specific lysis on mock-infected (mock) or pLJ-lacZ-, H5.CBALP-, and H5.CBlacZ-infected H2-compatible C57SV target cells in a 6-h ^{51}Cr release assay. Percentage of specific lysis is expressed as a function of different effector cell-to-target cell ratios (6:1, 12:1, 25:1, and 50:1).

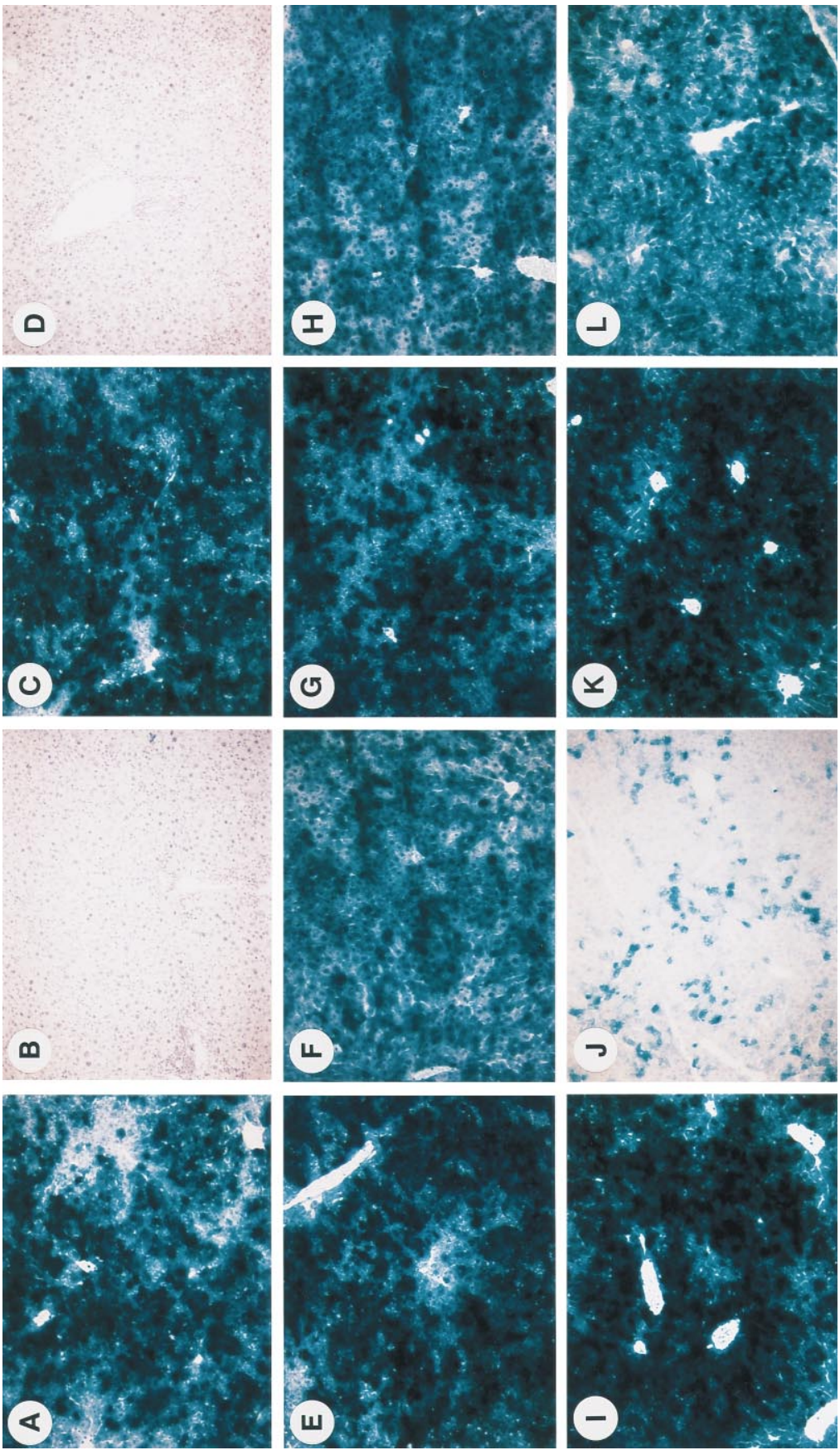

FIG. 7. Recombinant adenovirus-mediated transgene expression in mouse liver. Suspensions of virus (10^{11} particles) were infused into the tail veins of mice which were subsequently euthanized, and the liver tissues were evaluated for *lacZ* expression by X-Gal histochemistry 3 (first and third columns) and 60 (second and fourth columns) days later. First row, C57BL/6 mice infused with H5.CBlacZ (A and B) or H5.001CBlacZ (C and D); second row, *nu/nu* mice infused with H5.CBlacZ (E and F) or H5.001CBlacZ (G and H); and third row, ROSA-26 mice infused with H5.CBlacZ (I and J) or H5.001CBlacZ (K and L). Magnification, ×94.

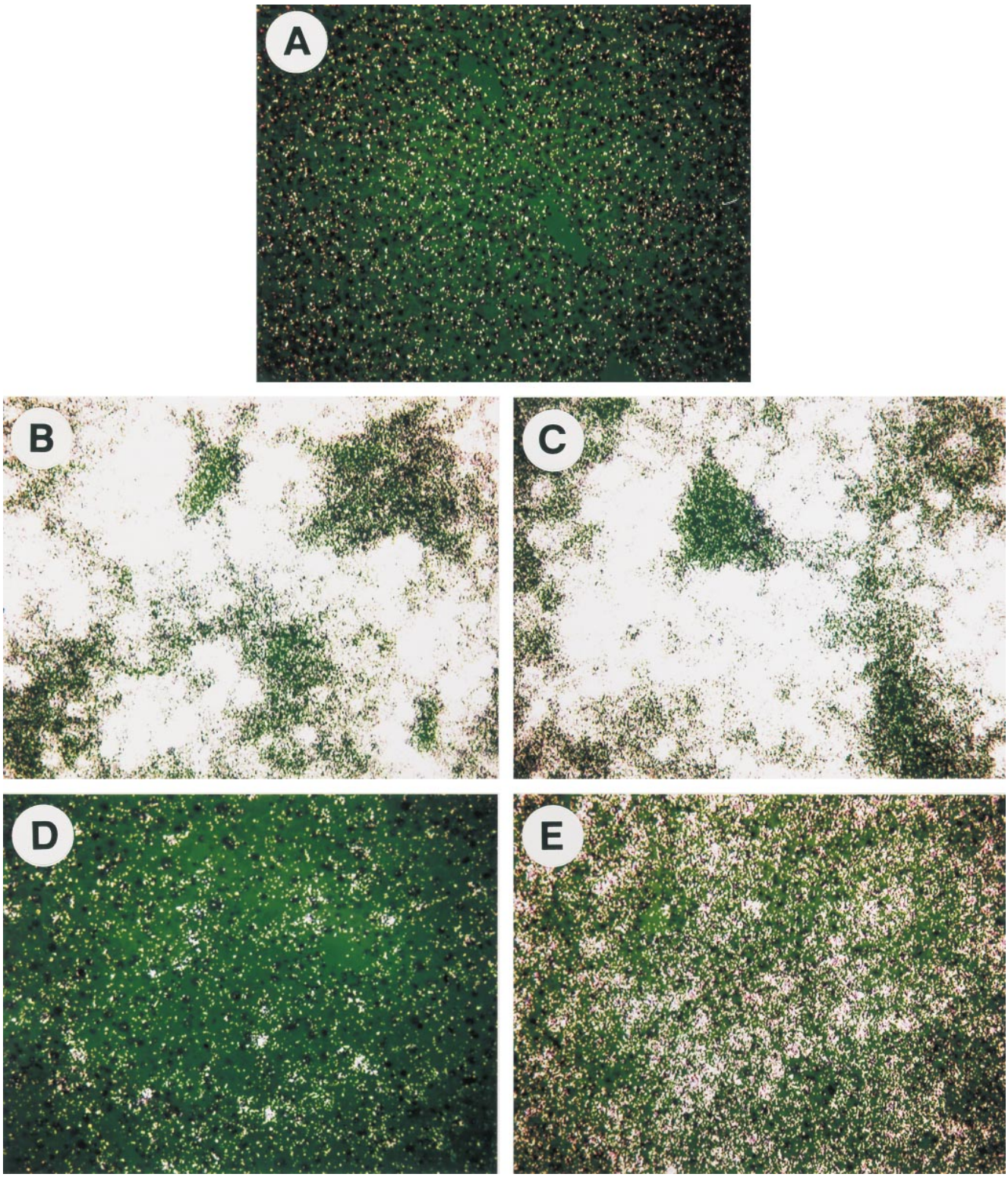

FIG. 8. Detection of recombinant adenovirus-derived *lacZ* expression in ROSA-26 mouse liver tissue by in situ hybridization. Naive ROSA-26 mice (A) and mice infected with H5.CBlacZ (B and D) or H5.001CBlacZ (C and E) were harvested 3 (B and C) or 60 (D and E) days later, and frozen sections were analyzed by in situ hybridization with RNA probes to simian virus 40 poly(A), which is unique to adenovirus-derived *lacZ* RNA. The slides were developed at day 10, and the results were analyzed by dark-field microscopy. Magnification, ×89.

TABLE 2. Morphometric analysis of mouse liver sections for transgene expression[a]

Mouse strain	% *lacZ* expression (mean ± 1 SD)			
	H5.CBlacZ		H5.001CBlacZ	
	Day 3	Day 60	Day 3	Day 60
C57BL/6	89.3 ± 2.4	0	92.4 ± 3.6	0
nu/nu	90.5 ± 1.5	82.3 ± 3.4	89.1 ± 4.5	80.4 ± 2.9
ROSA-26	93.4 ± 3.6	8.9 ± 4.2	91.8 ± 3.4	56.8 ± 3.7

[a] Data were quantified with Leica Quantimet 500$^+$ by analyzing a total of six sections from three mice for *lacZ*-expressing hepatocytes at days 3 and 60.

expression diminishing to less than 2-fold over 60 days (Table 2).

Analysis of ROSA-26 liver tissues by histochemistry as described above was consistent with a stabilizing effect of the E4 deletion, although a vector-induced activation of the endogenous *lacZ* transgene cannot be formally ruled out. An in situ hybridization assay was developed that discriminated between the germ line versus the vector-derived β-galactosidase mRNAs (Fig. 8). A cRNA probe was generated to noneukaryotic sequences uniquely represented in the 3′ untranslated region of the vector-encoded transcript. High-level hybridization was detected in ROSA-26 mice 3 days after injection of the E1-deleted (Fig. 8B) and the E1-E4-deleted (Fig. 8C) vectors, which was not present in uninjected ROSA-26 animals (Fig. 8A). Hybridization diminished over 60 days with both vectors, although significantly higher levels were observed with the E1-E4-deleted vector (Fig. 8D and E). In vitro studies were performed to document the role of CTLs to β-galactosidase in these models (Fig. 6). Low-level cytolysis to β-galactosidase-expressing targets from lymphocytes of C57BL/6 animals infected with either vector that was not present in ROSA-26 mice was demonstrated.

Implications for liver-directed gene therapy. The E1-E4-deleted vector has several advantages over a first-generation vector for liver-directed gene therapy. Analysis in murine models demonstrated clear safety advantages as assessed by histopathology and biochemical measures of liver damage such as serum transaminase levels. Expression of the vector-encoded transgene is prolonged when its product is eliminated as an immunologic target. Emergence of replication-competent adenovirus from a stock of E1-E4-deleted virus is unlikely, because it would require reversion at both E1 and E4; the latter event is highly unlikely, since there are no overlapping sequences between the transcomplementing cellular E4 gene and the *dl*1004-based E4 deleted vector.

The observation of diminished hepatotoxicity at day 14, when cell-mediated immunity dominates, was expected on the basis of the impact of the E4 deletion on viral protein expression. The doubly deleted vector was also less toxic at all time points in the athymic mouse and at day 3 in immune-competent mice before the onset of antigen-specific immunity. This suggests that viral gene expression may lead to direct toxicity. One mechanism may be the mobilization of nonantigen-specific immunity responses such as those mediated by NK cells (47). Alternatively, viral proteins may subvert important cellular processes that regulate cell viability and growth. An example would be the recently described effect of the E4 ORF6 on p53-mediated transcriptional activation (7). While this illustrates the role of ORF6 in fundamental cellular processes, this or other viral proteins likely have other effects because selective inhibition of p53 may actually prevent apoptosis. Either mechanism could lead to apoptosis-induced toxicity in the absence of antigen-activated immune effector cells. In general, the assessment of toxicity based on direct evaluation of histopathology agrees with the biochemical measurement of serum transaminase ALT levels.

There were striking differences in host responses in different strains of mice, with the most substantial pathology noted in C57BL/6 mice, in which inflammation dominated. This differed from the more blunted effects seen in C3H mice, in which apoptosis was the primary lesion. Strain-specific differences in the response of the host to the adenovirus vectors delivered to the liver have been described elsewhere (2). One contributing factor to these differences could be class I major histocompatibility complex-restricted variations in the presentation of vector antigens. We have recently shown that virus late gene products are dominant antigens in the *H-2*^{b} background of C57BL/6 mice, whereas the transgene dominates in the *H-2*^{k} background of C3H mice (17a). The efficient presentation of hexon proteins by the *H-2*^{b}-restricting allele products of C57BL/6 mice may diminish the advantages gained with the E1-E4-deleted vectors, by which late gene products are diminished but not eliminated; a combination of efficient antigen presentation with a low threshold of immune activation could explain the less substantive safety advantages afforded by the E1-E4 vectors in C57BL/6 mice.

Destructive immune responses to the transgene product β-galactosidase were documented in this model of liver-directed gene transfer that were similar to what has been described for adenovirus vectors delivered to the muscle (33, 38). In our studies, transgene-directed cellular responses were most evident when antigenic viral proteins were diminished by deleting both E1 and E4 in the vector. This illustrates the complexity of assessing vector biology when reporter genes are used that express neoantigens such as *E. coli* β-galactosidase, human alkaline phosphatase, or luciferase. Similar problems were encountered with gene replacement therapy in the mouse model of familial hypercholesterolemia (24).

We describe in this study consistent safety advantages of the E1-E4-deleted vector that, together with the diminished likelihood of replication-competent virus formation, suggest that it may be a preferred construct for applications of gene therapy that lead to liver-directed gene transfer. The recent observation that E4 ORF6 inhibited the tumor suppressor gene p53 further compels one to consider developing an adenovirus vector with E4 sequences deleted for gene therapy (7).

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